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(54) Title: POLYMERIC PEPTIDE PROBES AND USES THEREOF

(57) Abstract

An *in situ* hybridization assay for detection of rRNA or drug resistance gene(s) utilizing peptide nucleic acids (PNAs) or morpholino compounds as probes. Also provided are test kits useful for performing these assays.

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# POLYMERIC PEPTIDE PROBES AND USES THEREOF

## Background of the Invention

This invention relates generally to uncharged probes and more particularly, relates to the use of uncharged probes such as peptide nucleic acids (PNAs) and morpholinos in fluorescence *in situ* hybridization assays.

Current diagnostic methods in infectious diseases rely upon culturing infectious agents from patient test samples and subsequently identifying these infectious agents on the basis of microscopic morphology and staining characteristics, growth requirements, etc. Under current standard growth conditions, bacteria usually require 24 to 48 hours to grow, although some bacteria such as *Mycobacterium sp.* may require weeks to grow. After sufficient growth is attained, additional testing such as further biochemical tests, may be required to be performed in order to identify the bacteria. Some of these tests require an additional 18 to 48 hours before identification of the bacterium can be made. Concurrently with the biochemical tests, sensitivity tests may be performed in order to determine if the bacterium is susceptible to various antimicrobial agents. These methods rely on pure cultures of each bacterium. If a mixed culture is present, additional time is required to purify the bacteria present so that each can be identified and susceptibility tested.

Extensive research and development of fluorescence *in situ* hybridization (FISH) technology has occurred during the past decade. FISH routinely is applied to gene mapping, tumor characterization and prenatal diagnosis. See, for example, J. B. Lawrence et al., Science 249:928-932 (1990); P. Lichtner et al., Science 247:64-69 (1990); E. Viegas-Pequignot et al., Proc. Natl. Acad. Sci. USA 86:582-586 (1989); B. Trask et al., Genomics 5:710-717 (1989); H. Evans et al., Chromosoma 48:405-426 (1974); and D. C. Tkachuk et al., Genet. Anal. Tech. Appl. 8:67-74 (1991). Despite FISH's use in research and large medical center laboratories, it is not in widespread use in clinical laboratories. Its lack of use in clinical laboraratories probably is due to its lack of sensitivity, lack of standardized and user-friendly protocols, long turn-around time (usually, at least about 8 hours) and its manual, non-automated techniques, making it a labor-intensive and costly procedure to perform in the clinical laboratory.

It would be advantageous to provide an assay which would be able to detect less than 1000 copies of target nucleic acids or a single bacterial cell in a test

sample. It also would be advantageous to provide a simple, rapid FISH assay to detect and identify bacterial species in suspension or inside infected cells. Morover, it would be advantageous to provide an assay for detecting drug resistance genes in such bacterial species. Such detection could be performed simultaneously with identification. Such assays would be completed in eight hours or less and readily amenable to automation. It also would be advantageous to provide signal amplification options such that the sensitivity of FISH assays would be improved to the level required to provide clinically relevant results.

Summary of the Invention

An assay for detecting rRNA which may be present in a test sample is provided. The assay comprises the steps of contacting said test sample with a ppeptide nucleic acid (PNA) probe capable of attaching to said rRNA in said test sample conjugated to an indicator reagent comprising signal generating compound capable of generating a measurable signal; and detecting said measurable signal as an indication of the presence of rRNA in the test sample. The assay preferably is performed by flow cytometry. Quantitation is performed by exciting fluorescence and measuring said signal by using a light selection filter. The signal generating compound preferably is fluorescein or rhodamine. The rRNA in the test sample can be fixed prior to performing the assay. The assay further comprises hybridizing said test sample *in situ*.

The assay described hereinabove also can be performed using morpholino compounds as probes in place of the PNAs as probes. Also, an improved fluorescence *in situ* hybridization assay is provided wherein the improvement comprises hybridizing said test sample with a PNA or morpholino probe.

An assay for detecting drug resistance genes which may be present in a test sample also is provided herein. The assay comprises the steps of contacting said test sample which may contain drug resistance gene(s) with a peptide nucleic acid (PNA) probe or a morpholino probe capable of attaching to said drug resistance gene(s) in said test sample conjugated to an indicator reagent comprising signal generating compound capable of generating a measurable signal; and detecting said measurable signal as an indication of the presence of the drug resistance gene(s) in the test sample. The assay preferably is performed by flow cytometry. Quantitation is performed by exciting fluorescence and measuring said signal by using a light selection filter. The signal generating compound preferably is fluorescein or rhodamine. The drug resistance gene(s) in the test sample can be

fixed prior to performing the assay. The assay further comprises hybridizing said test sample *in situ*.

In addition, test kits are provided for detecting the presence of rRNA or drug resistance gene(s) which may be present in a test sample which comprise a container containing a PNA or morpholino probe conjugated to a signal generating compound capable of generating a measurable signal.

Brief Description of the Drawings

FIGURE 1 presents a histogram wherein a 25 mer DNA oligo and a 15 mer PNA oligo probe (sequence within the 25 mer of DNA sequence) complementary to 28S rRNA were directly labeled with fluorescein, wherein: A is a negative sample with DNA probe labeled at both ends and B is a positive sample with DNA probe labeled at both ends.

FIGURE 2 presents a histogram wherein a 25 mer DNA oligo and a 15 mer PNA oligo probe (sequence within the 25 mer of DNA sequence) complementary to 28S rRNA were directly labeled with fluorescein, wherein: C is a negative sample with PNA probe labeled at the amino end and and D is a positive sample with PNA probe labeled at the amino end.

FIGURE 3 presents a photograph of stained *E. coli* bacteria in mouse PMNs.

Detailed Description of the Invention

*In situ* hybridization was introduced in the late 1960's. J. G. Gall and M. Pardue, Proc. Natl. Acad. Sci. USA 63:378-383 (1969). Generally, it involves taking morphologically intact tissues, cells or chromosomes through the nucleic acid hybridization process to demonstrate not only the presence of a particular piece of genetic information but also its specific location within individual cells. It does not require the homogenization of cells and extraction of the target sequence, and therefore, provides precise localization and distribution of a sequence in cell populations. Secondly, the homogenization of tissues can result in a loss of sensitivity if the target is present in only a small fraction of the cells and at a low copy number. Such sequences would be difficult to detect in an extract because of a dilution effect caused by an excess of nontarget nucleic acids. *In situ* hybridization circumvents this problem by identifying the sequence of interest concentrated in the cells containing it. Thirdly, if a test sample contains heterogeneous cell populations, *in situ* hybridization methods can identify the type

and the fraction of the cells containing the sequence of interest. Finally, DNA as well as RNA can be detected with the same assay reagents.

FISH techniques have improved greatly during the past 15 years. The procedure has been simplified from its original tedious and laborious form. With the use of oligo probes, the assay has become more reproducible and easy to perform. For example, this type of assay can be done with multiple oligos in a one-step protocol that can be carried out in about two hours or less (J. Bresser et al., U. S. Patent No. 5,225,326. Attempts have also been made to automate *in situ* hybridization procedures. See, for example, C. Park et al., J. Histotechnology 14:219-229 (1991); E. R. Unger et al., J. Histotechnology 11:253-258 (1988). For example, Fisher Code-On™ (available from Fisher Scientific, Pittsburgh, PA) series slide stainer is capable of processing 60 microscopic slides through all of the steps required for *in situ* hybridization in a semi-automated fashion. In addition, Ventana Medical Systems, Inc. (Tucson, Arizona) recently introduced a completely automated *in situ* hybridization system capable of handling 40 slides. For FISH with cell suspensions and flow cytometric detection, the procedure also can be automated.

The intrinsic properties of *in situ* hybridization make it an extremely important tool in medical and clinical sciences; it has great potential in the clinical diagnosis of infectious diseases and cancer. Because only a small fraction (generally less than one in a hundred to a thousand) cells in tissues harbor viruses or bacteria, *in situ* hybridization is particularly useful in the analysis of infections. *In situ* hybridization, being an anatomic method of diagnosis, complements amplification-based assays such as PCR and LCR which are extremely sensitive but do not provide information on the cell types that are infected and quantitative information on the number of infected cells. *In situ* hybridization methods can determine the cells which are infected, the pathogen which is infecting the cells, and also it can determine (by quantitative measures) the extent of the infection. Labeled PNA probes directed against rRNA targets of microorganisms offer the potential to achieve the desired sensitivity and specificity with a practical protocol.

Among many potential detection schemes, fluorescence detection offers the fastest detection technology, the ability to multiplex and the potential for good quantitation in an automated format. We theorized that the combination of fluorescence detection techniques and *in situ* hybridization (fluorescence *in situ* hybridization or FISH) with so-called "peptide nucleic acids" ("PNA") probes targeting bacterial 16S rRNA, could be used to detect bacterial infection.

In the search for stable antisense agents, peptide nucleic acids ("PNAs") have emerged as useful probes for the recognition of single and double-stranded nucleic acids. M. Egholm et al., Nature 365:566-568 (1993). Synthetic PNA probes are polymeric analogs of peptide. The backbone of PNA is made from repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. Unlike natural peptides which have amino acids attached to the backbone, PNA contains purine (A, G) and pyrimidine (C, T) bases attached to the backbone by methylene carbonyl linkage. Unlike DNA or other DNA analogs, PNAs do not contain any pentose sugar moieties or phosphate groups. The molecules are neutral at physiological conditions. By convention, PNAs are depicted like peptides, with the N-terminus at the first (left) position and the C-terminus at the right. In addition to these chemical differences, PNA is biochemically different from both peptides and nucleic acids in that they are resistant to all known proteinases and nucleases. P.E. Nielsen et al., Science 254:1497-1500 (1991); M. Egholm et al., J. Am. Chem. Soc. 114, 1895-1897 (1992); M. Egholm et al., *ibid*, 114: 9677-9678 (1992); J. Hanvey et al., Science 258: 1481-1485 (1992); P.E. Nielsen et al., Nucleic Acids. Res 21:197-200 (1993); P. Matsudaira and J. Coull, ABRF News 4(3) (1993); H. Ørum et al., Nucleic Acids Res. 21:5332-5336 (1993). Despite these differences, PNAs have been shown to bind more strongly in solution to both DNA and RNA than the same length DNA oligonucleotides. The use of PNAs in *in situ* hybridization assays has not been demonstrated heretofore. Various factors have contributed to the uncertainty of the use of PNAs in *in situ* hybridization. For example, all cells have not only a large pool of variaty of macromolecules but also a very complex morphological structure. A probe that can be used successfully in fixed cells should have the ability to penetrate layers of cellular memberane under suitable conditions and, at the same time, should only bind to the designated target(s) but not to other components of a cell. We have discovered that fluorescently labeled PNA probes can be used for *in situ* hybridization, and that they provide remarkably improved (i.e., six times better) signal-to-noise than the corresponding DNA oligo. FIGURES 1 and 2 show a histogram of cell counts v. $\text{fluorescence}_{\log}$ wherein a 25 mer DNA oligonucleotide and a 15 mer PNA oligopeptide probe (sequence within the 25 mer of DNA sequence) complementary to 28S rRNA were directly labeled with fluorescein. The DNA oligonucleotide was labeled at both ends, while the PNA oligopeptide was labeled only at the amino end. When used in FISH with Chinese hamster ovary (CHO) cells and analyzed in flow cytometry, the one fluorophore PNA probe gave three times better linear

fluorescence signal to background than the two-fluorophore DNA probe. These data are discussed in detail in the examples provided hereinbelow. The advantages of using PNA probes are speed and the possibility of targeting short but specific regions of bacterial nucleic acids. Longer plasmid probes generally require overnight incubation, but 30 minutes to two hours is a sufficient time for short probes to penetrate cells and bind to the target. In addition, by carefully designing the probe sequence, PNA probes can be used as a general screen of all bacterial types when the probes are complementary to a consensus region of the target (so-termed "universal probe"). On the other hand, when the probes are designed to hybridize to sequence specific regions of a particular bacterial species, subtyping of bacteria can be achieved. Furthermore, the synthesis and labeling of probes are reproducible and the labelled probes are stable for years. Also, PNA probes are chemically very stable substituents. An additional advantage for using PNA probes is that PNAs are resistant to all known enzymes since they are not molecules which exist in nature.

FISH methods targeting rRNA for the identification of microbial cells were first introduced by DeLong et al., Science 243:1360-1363 (1989). Due to the abundance of rRNA ($10^4$ and $10^5$ copies per cell), phylogenetic identification of microbes was demonstrated by FISH using fluorescence microscopy and fluorescently labeled oligonucleotide probes (17-34 mers) complementary to bacterial 16S rRNA. In that study, it was shown that oligo probes could distinguish a bacterium dubbed "son-killer" (*Nasonia vitripennis*) and its closest known relative, *Proteus vulgaris*. These two organisms differ only slightly in size and shape and are difficult to distinguish using standard microbiological stains. Oligo probes labeled with two different fluorescent dyes easily identified one or the other bacterium in a mixed culture. These researchers also demonstrated that the rRNA content (and therefore the FISH signal) is directly proportional to the growth rate and metabolic activities of the microorganism. Similar studies utilizing a flow cytometer for fluorescence signal detection also have been documented (R. I. Amann et al., Applied and Environmental Microbiology 56:1919-1925 (1990); J. G. J. Bauman et al., J. Microscopy 157:73-81 (1989); Boye and Løbner-Olesen, The New Biologist 2:119-125 (1990); G. Wallner et al., Cytometry 14:136-143 (1993). In these studies, identification and quantitative analysis of mixed microbial populations were performed using fluorophore labeled oligonucleotide probes specific for 11 different organisms. Flow cytometric detection of yeast by *in situ*

hybridization with a fluorescent rRNA probe also has been demonstrated (B. Bertin et al., J. Microbiol. Methods 12:1-2 [1990]).

While we choose to take the advantages of *in situ* hybridization, other approaches for detecting and identifying microorganisms by targeting 16S rRNA have been documented. All of these methods applied DNA probes in a solution phase hybridization format with target rRNA extracted from cells. Some of these involved target amplification method such as polymerase chain reactions (PCR), ligase chain reaction (LCR) or nucleic acid sequence based amplification (NASBA). (Kohne, D. E. et al., US Patent No. 5,288,611; Greisen, K. et al., J. Clin. Microbiol. 32:335-351, (1994); Leong, D. U., European Patent No. EP 0 479 117 A1; Walker, G. T. et al., EP 0640 691 A2; Greisen, K. S. et al., International Patent NO. WO 93/03186 and Lane, D. J. et al., US patent No. 5401631A).

Recently, detection and identification of microorganisms inside infected cells has been demonstrated. Matsuhisa et al., Biotechnic and Histochemistry 69:31-37 (1994). In this work, enzymatically labeled genomic probes were used to detect and identify *Staphylococci* in mouse phagocytic cells. The technique has been extended to the diagnosis of bacteremia in the phagocytic cells of potentially septic patient blood samples. Matsuhisa et al., Microbiol. Immunol. 38:511-517, (1994). Current culture methods for detecting bacteria in blood samples can give false negative results and take two days to more than a week to obtain results, during which time a patient may die if administered treatment is not appropriate. The present invention provides an assay which has a fast turn around time and further provides information on the specific type of bacteria present. The assay disclosed herein also will work when more than one pathogenic species is present, as is detailed hereinbelow in the examples. Rapid identification of the bacterial species provides extremely valuble information for appropriate patient treatment.

The assay disclosed herein (PNA-FISH) potentially can be used for simultaneous identification of microorganisms and microbial drug resistance testing. Accompanying rapid developments in genome sequencing, more drug resistance genes are being identified and isolated, which allows the development of probe-based assay for direct detection of drug resistant strains at the genetic level. For review, see A. Linton et al., Schriftenr Ver Wasser Boden Lufthyg 78:197-224 (1988); J. T. Crawford et al., Respir. Infect. 9:62-70 (1994); and L.A. Anisimova et al., Mol. Gen. Mikrobiol. Virusol. (USSR) 11:3-12 (1988). In this assay, a hybridization cocktail could contain probes for microbial identification and probes targeting drug resistance genes or their transcripts (mRNA). Each of these probes

could be attached to a spectrally distinct fluorescent dye to allow multiparameter assay on one test sample.

The present invention also encompasses utilizing a capture reagent comprising a solid support having PNAs directly immobilized thereon which can can be contacted with a test sample, or, having the test sample directly immobilized on the solid support. The test sample can be any liquid suspected of containing a nucleic acid sequence which can specifically hybridize with the immobilized oligopeptides. The capture reagent can be contacted for a time and under conditions suitable for allowing nucleic acids in the test sample, if any, and the PNAs to hybridize and thereby form hybridization complexes. The hybridization complexes, if any, can be contacted with a conjugate for a time and under conditions sufficient to enable the conjugate to specifically bind any hybridization complexes. A signal can then be detected as an indication of the presence or amount of any nucleic acid sequences which may be present in the test sample.

Immobilized PNAs as taught herein can also be employed in a "one-step" assay configuration. According to such a configuration, a test sample suspected of containing nucleic acids which are complementary to the immobilized PNAs can be contacted with a conjugate for a time and under conditions suitable for allowing the conjugate to bind any nucleic acid sequences which may be present in the test sample to form conjugate/nucleic acid complexes. Alternatively, the nucleic acids which may be present in a test sample may comprise a detectable moiety. Nucleic acid sequences can be labeled or conjugated with a detectable moiety through, for example, nick translation whereby labeled nucleotides are incorporated into a target sequence. Conjugate/nucleic acid complexes or nucleic acids which comprise a detectable moiety can then be contacted with the support bound PNAs to form conjugate/nucleic acid/PNA complexes or nucleic acid/PNA complexes. A signal can then be detected as an indication of the presence or amount of any nucleic acid sequences present in the test sample.

In another embodiment, a method for quickly detecting the presence of an nucelic acids in a test sample is provided. According to this embodiment, a sample which is suspected of containing nucleic acids can be contacted with a support material and the nucleic acids which may be present in the test sample can be immobilized to the support material. A conjugate can then be contacted with the immobilized nucleic acids for a time and under conditions for allowing the conjugate to bind the immobilized nucleic acids. A signal generating compound

comprising PNAs can then be detected as an indication of the presence or amount of any nucleic acids which may have been present in the test sample.

The period for which nucleic acids which are immobilized as taught herein are contacted with, for example, a test sample, conjugate/nucleic acid complexes, or a conjugate is not important. However, it is preferred that such a contact period be kept to a minimum, for example, less than 30 minutes, more preferably less than 15 minutes and most preferably less than 10 minutes.

Those skilled in the art will understand that a conjugate may comprise a signal generating compound capable of generating a measurable signal attached to specific binding pair member . Signal generating compound (detectable moieties) may include any compound or conventional detectable chemical group having a detectable and measurable physical or chemical property variably referred to as a signal. Such detectable groups can be, but are not intended to be limited to, enzymatically active groups such as enzymes and enzyme substrates, prosthetic groups or coenzymes; spin labels; fluorescent molecules such as fluorescers and fluorogens; chromophores and chromogens; luminescent molecules such as luminescers, chemiluminescers and bioluminescers; phosphorescent molecules; specifically bindable ligands such as biotin and avidin; electroactive species; radioisotopes; toxins; drugs; haptens; polysaccharides; polypeptides; liposomes; colored or fluorescent particles; colored or fluorescent microparticles; colloidal particles such as selenium colloid or gold colloid; and the like. Additionally, a detectable moiety can comprise, for example, a plurality of fluorophores immobilized to a polymer such as that described in co-owned and co-pending U.S. Patent Application Serial No. 08/091,149 filed on July 13, 1993, which is herein incorporated by reference. The detectable physical or chemical property associated with a detectable moiety can be detected visually or by an external means. Specific binding member is a well known term and generally means a member of a binding pair, i.e., two different molecules where one of the molecules through chemical or physical means specifically binds to the other molecule. In addition to antigen and antibody specific binding pairs, other specific binding pairs include, but are not intended to be limited to, avidin and biotin, antibody and hapten, complementary nucleotide sequences or complementary nucleic acid sequences such as DNA or RNA, or PNAs, or morpholino compounds, an enzyme cofactor or substrate and an enzyme, a peptide sequence and an antibody specific for the sequence or an entire protein, dyes and protein binders, peptides and specific protein binders (e. g., ribonuclease, S-peptide and ribonuclease S-protein), and the like. Furthermore,

binding pairs can include members that are analogs of the original binding member, for example, an analyte-analog or a binding member made by recombinant techniques or molecular engineering. Thus, PNAs and morpholino compounds are specific binding members for DNA or RNA. If the binding member is an immunoreactant it can be, for example, a monoclonal or polyclonal antibody, a recombinant protein or recombinant antibody, a chimeric antibody, a mixture(s) or fragment(s) of the foregoing. Signal generating compounds can be attached to specific binding pair members through any chemical means and/or physical means that do not destroy the specific binding properties of the specific binding member or the detectable properties of the detectable moiety.

A method provided herein can be employed to immobilize oligons to a glass surface which is then employed in further analysis, such as a waveguide configuration as that taught in co-owned and co-pending U.S. Patent Application Serial No. 08/311,462 entitled "Light Scattering Optical Waveguide Method for Detecting Specific Binding Events" which is herein incorporated by reference. A waveguide device's ability to be employed in an immunoassay type format is based upon a phenomenon called total internal reflection (TIR). TIR operates upon the principle that light traveling in a denser medium (i.e. having the higher refractive index, $N_1$) and striking the interface between the denser medium and a rarer medium (i.e. having the lower refractive index, $N_2$) is totally reflected within the denser medium if it strikes the interface at an angle, $q_R$, greater than the critical angle, $q_C$, where the critical angle is defined by the equation:

$$\theta_C = \arcsin\left(N_2/N_1\right)$$

Under these conditions, an electromagnetic waveform known as an "evanescent wave" is generated. The electric field associated with the light in the denser medium forms a standing sinusoidal wave normal to the interface. The evanescent wave penetrates into the rarer medium, but its energy E dissipates exponentially as a function of distance Z from the interface. A parameter known as "penetration depth" ($d_p$) is defined as the distance from the interface at which the evanescent wave energy has fallen to 0.368 times the energy value at the interface. [See, Sutherland et al., J. Immunol. Meth., 74:253-265 (1984) defining $d_p$ as the depth where $E=(e^{-1})\cdot E_0$]. Penetration depth is calculated as follows:

$$d_p = \frac{\lambda/N_1}{2\pi\left\{\sin^2\theta_R - (N_2/N_1)^2\right\}^{1/2}}$$

Factors that tend to increase the penetration depth are increasing angle of incidence, $\theta_R$; closely matching indices of refraction of the two media (i.e.

$N_2/N_1$ --> 1); and increasing wavelength, $\lambda$. For example, if a quartz TIR element ($N_1$ = 1.46) is placed in an aqueous medium ($N_2$ = 1.34), the critical angle, $\theta_C$, is 66° (= arcsin 0.9178). If 500 nm light impacts the interface at $\theta_R$ = 70° (i.e. greater than the critical angle) the $d_p$ is approximately 270 nm.

TIR has also been used in conjunction with light scattering detection in a technique referred to as Scattered Total Internal Reflectance ("STIR"). See, e.g., U.S. Patents 4,979,821 and 5,017,009 to Schutt, et al and WO 94/00763. According to this technique, a beam of light is scanned across the surface of a TIR element at a suitable angle and the light energy is totally reflected except for the evanescent wave. Particles such as red blood cells, colloidal gold or latex specifically bound within the penetration depth will scatter the light and the scattered light is detected by a photodetection means.

Immobilizing oligos contained to support materials according to the present invention comprises contacting a support material with an oligo solution and drying the solution upon the support material. If the oligo is suspected of being contained within a test sample, the test sample is dried upon the support material.

Support materials or solid supports (so-termed "solid phases") to which oligos can be immobilized are well known in the art and include materials that are substantially insoluble. Porous materials can serve as solid supports and may include, for example, paper; nylon; and cellulose as well as its derivatives such as nitrocellulose. Smooth polymeric and nonpolymeric materials are also suitable support materials and include, but are not intended to be limited to, plastics and derivatized plastics such as, for example, polycarbonate, polystyrene, and polypropylene; magnetic or non-magnetic metal; quartz and glass. Preferably, quartz, glass or nitrocellulose is employed as a support material. Solid supports can be used in many configurations well known to those skilled in the art including, but not limited to, test tubes, microtiter wells, sheets, films, strips, beads, microparticles, chips, slides, cover slips, and the like.

Oligonucleotides according to the invention will be understood to mean a sequence of DNA or RNA, whereas the term oligopeptides will be understood to mean a sequence of PNA or morpholino compounds. All may be generally termed as oligos herein. Both PNAs and morpholino compounds have a higher binding affinity, better penetrability and lower susceptibilityt o enzymatic digestion than nucleic acid probes. The length of an oligo which is immobilized to a support material is largely a matter of choice for one skilled in the art and is typically based upon the length of a complementary sequence of, for example, DNA, RNA, or

PNA or morpholino compound which will be captured. While the length of an immobilized oligo is typically between about 5 and about 50 base pairs, preferably, the length of an immobilized oligo is between about 5 and about 30 base pairs, more typically between about 10 and about 25 base pairs.

A "capture reagent", as used herein, refers to an unlabeled specific binding member which is specific either for the analyte as in a sandwich assay, for the indicator reagent or analyte as in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample .

Test samples which can be tested by the methods of the present invention described herein include human and animal body fluids which can contain nucleic acids such as whole blood, serum, plasma, cerebrospinal fluid, urine, biological fluids such as cell culture supernatants, fixed tissue specimens and fixed cell specimens. It also is within the scope of the present invention that a variety of non-human or non-animal body fluids which can contain nucleic acids also can be analyzed according to the present invention.

Synthesis of oligos is routine using automated synthesizers. If desired, automated synthesizers can produce oligoss which are modified with terminal amines or other groups. A useful review of coupling chemistries is found in Goodchild, Bioconjugate Chemistry, 1(3):165-187 (1990).

The amount of oligo solution (which can be a test sample) that is applied or "spotted" upon a solid support need be large enough only to capture sufficient complementary sequences to enable detection of, for example, a captured sequence or conjugate. This is dependent in part on the density of support material to which the capture oligo is immobilized. For example, areas of as little as 150 μm in diameter may be employed. Such small areas are preferred when many sites on a support material are spotted with oligonucleotide solution(s). The practical lower limit of size is about 1μm in diameter. For visual detection, areas large enough to be detected without magnification are desired; for example at least about 1 to about 50 $mm^2$; up to as large as 1 $cm^2$ or even larger. There is no upper size limit except as dictated by manufacturing costs and user convenience.

Once an oligo solution is contacted with a solid support, evaporation is the preferred drying method and may be performed at room temperature (about 25°C). When desired, the evaporation may be performed at an elevated temperature, so

long as the temperature does not significantly inhibit the ability of the oligos to specifically hybridize with complementary sequences.

The process of immobilizing oligos to a solid support may further comprise "baking" the support material and the oligo solution thereon. Baking may include subjecting the solid phase and oligonucleotide solution residue, to temperatures between about 60°C and about 95°C, preferably between about 70°C and about 80°C. The baking time is not critical and preferably lasts for between about 15 minutes and about 90 minutes. Baking is particularly preferred when porous support materials such as, for example, nitrocellulose are employed.

An overcoating step may optionally be employed in the method herein provided. Overcoating typically comprises treating the support material so as to block non-specific interactions between the support material and complementary sequences which may be in a fluid sample. It is preferred that the overcoating or blocking material is applied after the oligo solution has been dried upon the support material. In cases where a baking step is employed, the blocking material should be applied after the baking step. Suitable blocking materials are casein, zein, bovine serum albumin (BSA), 0.5% sodiumdodecyl sulfate (SDS), and 1X to 5X Denhardt's solution (1X Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.2 mg/ml BSA). Other blockers can include detergents and long-chain water soluble polymers.

Casein has been found to be a preferred blocking material and is available from Sigma Chemical, St. Louis, MO. Casein belongs to a class of proteins known as "meta-soluble" proteins (see, e.g., U.S. Patent 5,120,643 to Ching, et al, incorporated herein by reference) which are preferably treated to render them more soluble. Such treatments include acid or alkaline treatment and are believed to perform cleavage and/or partial hydrolysis of the intact protein. Casein is a milk protein having a molecular weight of about 23,600 (bovine beta-casein), but as used herein, "casein" or "alkaline treated" casein both refer to a partially hydrolyzed mixture that results from alkaline treatment as described in example 1 of US Patent 5,120,643. An electrophoresis gel (20% polyacrylamide TBE) of the so-treated casein shows a mixture of fragments predominantly having molecular weight less than 15,000, as shown by a diffused band below this marker.

A preferred assay method for the detection of nucleic acids in a test sample according to the present invention includes flow cytometric procedures and particle counting procedures. For example, in particle counting, analytes which are members of specific binding pairs are quantified by mixing an aliquot of test sample

with microparticles coated with a capture reagent capable of binding to the nucleic acid of interest as the other member of the specific binding pair. If the nucleic acid is present in the test sample, it will bind to some of the microparticles coated with the capture reagent and agglutinates will form. The analyte concentration is inversely proportional to the unagglutinated particle count. See, for example, Rose et al., eds., Manual of Clinical Laboratory Immunology, 3rd edition, Chapter 8, pages 43-48, American Society for Microbiology, Washington, D. C. (1986).

Flow cytometry methods that sense electronic and optical signals from cells which are illuminated allows determination of cell surface characteristics, volume and cell size. Nucleic acids present in, for example, bacteria present in a test sample are bound to the PNA or comorpholino compound and detected with a fluorescent dye which is either directly conjugated to the PNA or morpholino compound or added via a second reaction. Different dyes, which may be excitable at different wavelengths, can be used with more than one PNA or morpholino compound specific to different nucleic acids such that more than one type of nucleic acid can be detected from one sample. In fluorescence flow cytometry, a suspension of particles, typically cells in a test sample, is transported through a flowcell where the individual particles in the sample are illuminated with one or more focused light beams. One or more detectors detect the interaction between the light beam(s) and the labeled particles flowing through the flowcell. Commonly, some of the detectors are designed to measure fluorescence emissions, while other detectors measure scatter intensity or pulse duration. Thus, each particle that passes through the flowcell can be mapped into a feature space whose axes are the emission colors, light intensities, or other properties, i.e., scatter, measured by the detectors. In one situation, the different particles in the sample map into distinct and non-overlapping regions of the feature space, allowing each particle to be analyzed based on its mapping in the feature space. To prepare a test sample for flow cytometry analysis, the operator manually pipettes a volume of test sample from the sample tube into an analysis tube. A volume of the desired fluorochrome labeled PNA or morpholino compound is added. The sample/PNA or morpholino compound mixure then is incubated for a time and under conditions sufficient to allow nucleic acid/PNA or morpholino compound bindings to take place. After incubation, and if necessary, the operator adds a volume of RNS lyse to destroy any RBCs in the sample. After lysis, the sample is centrifuged and washed to remove any left-over debris from the lysing step. The centrifuge/wash step may be repeated several times. The sample is resuspended in a volume of a fixative and the

sample then passes through the fluorescence flow cytometry instrument. A method and apparatus for performing flow automated analysis is described in co-owned U.S. Patent application Serial No. 08/283,379, which is incorporated herein by reference. It is within the scope of the present invention that microspheres can be utilized in the methods described herein, tagged or labeled, and employed for *in vitro* diagnostic applications. It also is within the scope of the present invention that other cells or particles, including bacteria, viruses, durocytes, etc., can be tagged or labeled with the PNAs or morpholino compound as described by the present invention and used in flow cytometric methods.

It is contemplated that the reagent employed for the *in vitro* assays can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a PNA or morpholino compound, or a cocktail of these compounds, employed in the assay(s). These kits also could contain vials or containers of other reagents needed for performing the assay(s), such as washing, processing and indicator reagents.

The present invention will now be described by way of examples, which are meant to illustrate, but not to limit, the spirit and scope of the invention.

## EXAMPLES

For convenience only, the Sequence Listing provided herein contains a listing of both DNA and PNA probes. The PNA sequences in the Sequence Listing, denoted as SEQUENCE I.D. NOS. 4 through 9, are stated in the Sequence Listing as having fluorescein labelling at the 5' end of the molecule, and are denoted as DNA. Actually, the amino-terminal end of the PNA molecule was labelled with fluorescein. Also, the molecule in SEQUENCE I.D. NOS. 4 through 9 are PNA molecules, and not DNA molecules. The amino end of the PNA molecule was termed the 5' end in the Sequence Listing but is not considered as the 5' end of an oligopeptide. A PNA molecule is not a DNA genomic molecule.

### Example 1. Comparison of FISH and DNA Probe Hybridization Efficiency

A. Experimental Protocol. PNA and DNA probe hybridization efficiencies were compared using protocols and hybridization conditions optimized for DNA. Detection was by an EPICS®Profile II (Coulter Corp., Hialeah, Florida) flow cytometer equipped with an Argon laser. The laser was set to 15mW at 488nm.

Fluorescein fluorescence was acquired using the light selection filter of 525/30 nm (central wavelength/full bandwidth at half-maximal transmission).

In this experiment, the flourescent signal intensity of the PNA (SEQUENCE I.D. NO. 4) and DNA probes (SEQUENCE I.D. NO. 1) were compared. The DNA oligo probe (SEQUENCE I.D. NO. 1) was a 25mer that amplified 28S rRNA of mammalian cells. It was labeled with fluorescein at both the 3' and 5' ends. The PNA probe (SEQUENCE I.D. NO. 4) was a 15mer whose sequence resided within that of (SEQUENCE I.D. NO. 1). SEQUENCE I.D. NO. 1 was labeled with fluorescein only at the amino-end of the polypeptide. The negative control DNA probe (SEQUENCE I.D. NO. 2) was a 25mer complimentary to pBR322 sequence and labeled in the same manner as SEQUENCE I.D. NO. 1. The negative control PNA probe (SEQUENCE I.D. NO. 5) was a 15mer complimentary to Hepatitis B viral DNA (positions 330-344). It was labeled in the same manner as SEQUENCE I.D. NO. 4.

B. Cell Fixation. Chinese Hamster Ovary (CHO) cells (A.T.C.C. No. CRL 9618, available from the American Type Culture Collection [A.T.C.C.], 12301 Parklawn Drive, Rockville, MD 20852) were grown in F12 medium, supplemented with 10% serum (Cat. No. 16140, Life Technology, Grand Island, NY). The cells were collected by trypsinization and centrifugation at 450 x g for 10 min (IECCentra-8R Centrifuge, International Equipment Co., Needham Hts., MA). The cells were washed with PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) twice and fixed immediately with 4% paraformaldehyde for 15 min at room temperature. The cells then were washed twice with PBS and stored either in PBS at 4°C for up to a week or in 70% ethanol for months at -20°C.

C. *In Situ* Hybridization. Cells were incubated in 0.01% pepsin in 0.02 N HCl for 10 min at 37°C, washed once with PBS + 0.02% glycine for 2 min and finally washed twice more with PBS. The cells were post-fixed with 2% paraformaldehyde for 5 min at room temperature, washed with PBS once and then resuspended in HBSS (0.14 M NaCl, 5.4 mM KCl, 0.7 mM $NaHPO_4$, 1 mM $NaHCO_3$, pH 7.3). One volume of 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 2 volumes of formamide were added to the cells, and then they were prehybridized at room temperature for at least 10min. Meanwhile, probes (20 pmol PNA [SEQUENCE I.D. NO. 4 ] or DNA [SEQUENCE I.D. NO. 1] per million cells) were resuspended in 15 µl hybridization mixture (2X SSC, 50% formamide, 0.5% SDS, 100 µg/ml Salmon sperm DNA [available from Boehringer Mannhein,

Indianapolis, IN) to form a probe mixture. The probe mixture was heated in a boiling water bath for 2 min and cooled down rapidly on ice. Cells were spun down at 450 x g for 10 min and the probe mixture was hybridized to the cells. Probes then were hybridized for 3 hours at 40°C. The cells were washed first with 2 X SSC, 50% formamide and 0.5% SDS, then 2X SSC with 0.1X SSC, each for 30 min at 40°C. The cells which were resuspended in PBS were analyzed by flow cytometry.

D. Results. Stained cells were analyzed by flow cytometry (EPICS®Profile II, Coulter Corp., Hialeah, Florida), equipped with an Argon laser. Average linear fluorescence signals of the negative control cells and the positive samples are listed below in TABLE 1. The results clearly showed that the PNA probe (SEQUENCE I.D. NO. 4 ), although much shorter in length, provided a higher signal to noise ratio compared to the corresponding DNA probe (SEQUENCE I.D. NO. 1 ). The PNA probe (SEQUENCE I.D. NO. 4) provided approximately six times higher signal to noise ratio, considering that the PNA probe (SEQUENCE I.D. NO. 4 ) had only one fluorescent label as compared to the DNA probe (SEQUENCE I.D. NO. 1 ) which had two.

TABLE 1

| Probe Type | Neg. Ctrl. Probe | 28S rRNA Probe | Signal to Noise |
|---|---|---|---|
| DNA 25mer-2FL | 0.209 | 3.295 | 15.8 |
| (SEQ I.D. NO. 1) | (SEQ I.D. NO.2.) | (SEQ I.D. NO.1 ) | |
| PNA 15mer-1FL | 0.331 | 15.18 | 45.9 |
| (SEQ I.D. NO. 4) | (SEQ I.D. NO.5 ) | (SEQ I.D. NO. 4) | |

Example 2. Ionic Strength Comparison

A. Experimental Protocol. In this experiment, different salt buffers in the hybridization cocktail were tested using CHO cells as described in Example 1. The FISH protocol as described in Example 1 was followed. Twenty (20) pmol of the EuB338 probe (SEQUENCE I.D. No.7 ) was dissolved per cell sample in various hybridization cocktails for comparison. A negative control sample (treated with 100 µg/ml RNase A [available from Sigma, St. Louis, MO] in PBS at room temperature for 1 hour) was included for each hybridization cocktail tested.

The compositions of hybridization cocktails compared were (1) B1: 2X SSC, 50% formamide (Molecular Biology Grade, available from Fisher Scientific, Pittsburgh, PA), 0.5% SDS (available from Sigma, St. Louis, MO) and 0.1% BSA (available from Sigma, St. Louis, MO); (2) B2: PBS, 50% formamide, 0.5% SDS and 0.1% BSA; (3) B3: TE (10 mM Tris, 1 mM EDTA), 50% formamide, 0.5% SDS and 0.1% BSA.

B. Results. The stained samples were analyzed by flow cytometry as described in Example 1. Average fluorescence intensity of negative control and positive samples are shown in TABLE 2. The signal to noise ratio was defined as the fluorescence intensity of positive sample divided by that of the corresponding negative control.

TABLE 2

| Samples | Fluoresc. Intensity of Neg. Control | Fluoresc. Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| B1 | 0.306 | 5.064 | 16.3 |
| B2 | 0.403 | 6.555 | 16.4 |
| B3 | 0.982 | 6.501 | 6.8 |

The results as shown above in TABLE 2 demonstrate that the fluorescence signals of PNA hybridization to the target RNA was not dependent on the salt buffer used in the hybridization cocktail. In TE (10mM Tris.Cl, 1mM EDTA, pH 7.4) buffer, however, nonspecific binding of the probe to the target was much greater than that in SSC or PBS buffer. This was reflected in a reduced signal to noise ratio. However, PBS and SSC buffers had equivalent signal to noise of about 16. The SSC buffer is commonly used in fluorescence *in situ* hybridization with DNA probes.

## Example 3. Evaluation of Denaturing Reagents

This example was designed to determine the optimal concentration of denaturing reagents for PNA *in situ* hybridization.

A. Cell Fixation. Briefly, *E. coli* (available from the A.T.C.C., 12301 Parklawn Drive, Rockville, MD 20852 as ATCC deposit number 8739) was grown in trypticase soy broth (TSB, available from DIFCO Laboratories, Detroit, MI) at

35°C overnight. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells next were washed with PBS twice and stored in PBS at 4°C for up to a week.

B. *In Situ* Hybridization. *E. coli* cells in PBS ([A.T.C.C. deposit number 8739] 1ml per sample, $10^9$ cells/ml), prepared as described in this Example hereinabove, were pelleted by centrifugation at 1000 x g for 10 min. The cells were resuspended in 20 mM Tris + 2 mM $CaCl_2$. Proteinase K was added to 1 µg/ml and incubated at 37°C for 7.5 min. The cells then were washed with PBS + 0.02% glycine, followed by two more washes with PBS. For negative control samples, *E. coli* cells in PBS were incubated with 100 µg/ml RNase A for 1 hour at room temperature. The cells then were washed twice with PBS. All samples were spun down and resuspended in 100 µl of hybridization cocktail without probe and allowed to pre-hybridize for 10 min at room temperature. The cells next were pelleted, and 20 µl hybridization cocktail with 20 pmol of Probe 7 (SEQUENCE I.D. NO. 7 ) was added to the cell pellet and allowed to hybridize for 3 hours at 38°C.

Hybridization cocktails with 50% formamide (standard for DNA hybridization), various concentrations of urea or no denaturing agents were tested by the methods described herein. The compositions of various hybridization cocktails are shown in TABLE 3.

TABLE 3

| BUFFER DESIGNATION | COMPOSITION |
|---|---|
| B0 | PBS+10% DMSO (Sigma) + 0.5%SDS + 0.1% BSA --- NO DENATURANT |
| B1 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA + 50% foramide |
| B2 | PBS + 10% DMSO + 0.5% SDS+ 0.1% BSA + 0.25M urea |
| B3 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA + 0.5M urea |

TABLE 3 (CONTINUED)

| BUFFER DESIGNATION | COMPOSITION |
|---|---|
| B4 | PBS + 10% DMSO + 0.5% SDS + 0.1%BSA + 1M urea |
| B5 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA + 2M urea |
| B6 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA + 4M urea |
| B7 | PBS + 10% DMSO + 0.5% SDS + 0.1%BSA + 8M urea |

C. Results. After hybridization, cells were analyzed on the same flow cytometer described in the previous examples. The average fluorescence intensities of negative controls and positive samples were recorded and are presented in TABLE 4. Signal to noise (S/N) were calculated as described previously in Example 2.

TABLE 4

| Samples | Fluores. Intensity of Neg. Control | Fluoresc.Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| B0 | 0.108 | 1.365 | 12.6 |
| B1 | 0.107 | 1.347 | 12.6 |
| B2 | 0.107 | 1.159 | 10.8 |
| B3 | 0.109 | 1.127 | 10.3 |
| B4 | 0.107 | 1.238 | 11.6 |
| B5 | 0.109 | 1.083 | 9.9 |
| B6 | 0.111 | 0.867 | 7.8 |
| B7 | 0.123 | 1.078 | 8.8 |

The results shown above in TABLE 4 demonstrate that the PNA hybridization signal was not affected by denaturing agents such as 50% formamide. This hybridization is commonly used for DNA probe in situ hybridization.

However, urea, especially at high concentrations, weakened the hybridization between the PNA probe (SEQUENCE I.D. NO. 7) and the RNA target.

As is known in the art, the purpose of using denaturing agent in DNA probe hybridizations is to make the target accessible to the probe. The targets, double stranded or single stranded but with complicated secondary structure, have low hybridization efficiencies if denaturing agents such as formamide are not used. PNAs, however, have a much stronger binding efficiency to the target and can unwind double stranded DNA upon strand-displacement (Egholm, M. et al., J. Am. Chem. Soc. 114:9677-9678, (1992); Cherny, D. Y. el al., Proc. Natl. Acad. Sci. U. S. A. 90:1667, (1993)). These data demonstrate that denaturing agents for improving probe accessibility can be eliminated when utilizng PNAs, as a comparison of hybridization signal to noise ratios (S/N) of sample B0 and B1 (TABLE 4) clearly demonstrate. It thus was not surprising that the hybridization signal decreased as the concentration of denaturing agent increased, since denaturing agent such as urea can disrupt hydrogen bonds, the primary force for the hybridization of PNA to DNA and RNA.

Example 4. Effect of Other Components in the Hybridization Buffer

A. Experimental Protocol: In this experiment, hybridization buffer conditions were optimized and simplified for DNA probes. Conditions tested included a permeation reagent (DMSO), a detergent (SDS), a non-specific binding blocking reagents (BSA and Salmon sperm DNA). The following buffer conpositions used are presented in TABLE 5.

TABLE 5

| BUFFER DESIGNATION | COMPOSITION |
|---|---|
| B1 | PBS |
| B2 | PBS +10% DMSO |
| B3 | PBS + 0.5% SDS |
| B4 | PBS + 10% DMSO + 0.5% SDS |
| B5 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA |
| B6 | PBS + 10% DMSO + 0.5% SDS + 0.1% BSA + 100ug/ml Salmon sperm DNA |

Eub338 probe (SEQUENCE I. D. NO 7), dissolved in different compositions described in TABLE 5 was added to *E. coli* and the negative controls (*E. coli* treated with RNase). After hybridization as described in Experiment 3, samples were analyzed on flow cytometer.

B. Results:

The results of the above-described experiment are summarized below in TABLE 6.

TABLE 6

| Buffer | Fluorescence of Neg. Control | Fluorescence of positive sample | Signal-to-Noise |
|---|---|---|---|
| B1 | 0.109 | 0.124 | 1.14 |
| B2 | 0.107 | 0.127 | 1.19 |
| B3 | 0.106 | 1.273 | 11.98 |
| B4 | 0.106 | 1.315 | 12.41 |
| B5 | 0.106 | 1.085 | 10.23 |
| B6 | 0.106 | 0.808 | 7.62 |

The data from TABLE 6 shows that SDS is a critical factor in the hybridization. Other commonly used components in a typical DNA-FISH assay, such as DMSO and BSA, did not significantly affect the hybridization. Salmon sperm DNA is used in DNA-FISH hybridization cocktails to block positively charged cellular components. Since PNAs are neutral, it was hypothesized that such blocking of DNA may not be needed in the hybridization cocktail for PNAs. Our data summarized hereinabove in TABLE 6 confirmed this hypothesis.

## Example 5. Effect of Detergents in Hybridization Buffer

A. Experimental Protocol. This experiment compares the effect of different detergents in the hybridization buffer. E. coli cells were grown, fixed and hybridized to Eub338 probe (Sequence I.D. NO. 7) as described in Example 3. The compositions of various hybridization cocktails are shown in TABLE 7.

TABLE 7

| BUFFER DESIGNATION | COMPOSITION |
|---|---|
| B1 | PBS+0.1%SDS |
| B2 | PBS + 0.5% SDS |
| B3 | PBS + 0.1% Triton X-100 |
| B4 | PBS + 0.5% Triton X-100 |
| B5 | PBS + 0.1% Tween-20 |
| B6 | PBS + 0.5% Tween-20 |

B. Results:

After hybridization, the cells were analyzed on the same flow cytometer. The average fluorescence intensities of negative controls and positive samples were recorded and are presented in TABLE 8. Signal to noise (S/N) was calculated as described previously in Example 2).

TABLE 8

| Samples | Fluores.Intensity of Neg. Control | Fluoresc.Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| B1 | 0.106 | 1.975 | 18.6 |
| B2 | 0.131 | 2.118 | 16.2 |
| B3 | 0.112 | 0.263 | 2.34 |
| B4 | 0.131 | 0.301 | 2.3 |
| B5 | 0.0.110 | 0.268 | 2.44 |
| B6 | 0.116 | 0.266 | 2.29 |

The data presented in TABLE 8 clearly shows that the PNA probe did not bind efficiently to the target rRNA in the presence of commonly used detergents in DNA-FISH assays such as Triton X-100® or Tween-20®. Thus, SDS must be included in the hybridization buffer to provide probe accessibility.

Example 6. Application of PNA FISH Hybridization to Alternative Targets

A. Experimental Protocol. In this experiment, two types of bacteria (two strains of the yeast *Saccharomyces Cerevisiae* (strain YJO, obtained from Dr. B. Kohorn,

Dept. of Botany, Duke University, Durham, NC., PNAS 88:5159-5162, [1991] and strain E8-11C, obtained from Dr. E. T. Young, Univ. of Washington, Seattle, WA), and a gram-positive bacteria *Staphylococcus aureus*, ATCC 6538, obtained from the A.T.C.C., 12301 Parklawn Drive, Rockville, Maryland 20852) were hybridized to the 16S rRNA universal probe (SEQUENCE I.D. NO. 6 ), as follows, to demonstrate the universality of this PNA FISH approach. RNase treated samples were used as negative controls.

Yeast cells were grown in yeast peptone dextrose (YPD, DIFCO Laboratories, Detroit, MI) medium overnight at 29°C and *S. aureus* was grown in trypticase soy broth medium (TSB) (DIFCO Laboratoreis, Detroit, MI) overnight at 35°C. The cells were collected by centrifugation at 2500 rpm for 8 min and washed with PBS three times. Cells were fixed and treated as described in Example 3. One (1) x $10^8$ cells were used in each sample. Twenty (20) pmol of the universal probe (SEQUENCE I.D. NO. 6 ) which hybridized to all 16S like rRNA was dissolved in hybridization buffer (PBS + 0.5% SDS), applied to each sample ($10^8$ cells) and allowed to hybridize. After post-hybridization washes with PBS, the cells were analyzed as described in Example 3.

B. Results. The results were summarized in TABLE 9. The data in TABLE 9 demonstrated that the PNA *in situ* hybridization protocol developed in this study can be generalized to a gram-positive bacterium and yeast .

TABLE 9

| Samples | Fluoresc. Intensity. of Neg. Control | Fluoresc. Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| *S. cerevisiae* YJO | 0.106 | 0.963 | 9.08 |
| *S. cerevisiae* E8-11C | 0.322 | 2.118 | 6.58 |
| *S. aureus* | 0.109 | 0.39 | 3.58 |

Example 7. PNA Probe Specificity

A. Experimental Protocol. This experiment tested the specificity of the PNA *in situ* hybridization protocol developed in Example 1. Five (5) different probes were applied to *E. coli*. (ATCC deposit number 8739, obtained as described hereinabove). The simplified and optimized protocol developed for PNA and

described in Example 3 was followed. TABLE 10 presents the probes used in the study.

TABLE 10

| PROBE | DESCRIPTION |
|---|---|
| Probe 4 (SEQUENCE I.D. No. 4) | complementary to mammalian 28S rRNA (position 1901-1915) |
| Probe 9 (SEQUENCE I.D. No. 9) | complementary to S. Aureus 16S rRNA found only in (position 78-93) |
| Probe 6 (SEQUENCE I.D. No. 6) | complementary to the conserved region of microbial 16S rRNA (position 1392-1406) |
| Probe 7 (SEQUENCE I.D. No. 7) | complementary to conserved region of eubacterial 16S rRNA (338-352) |
| Probe 8 (SEQUENCE I.D. No. 8) | complementary to conserved region of eukaryotic 16S rRNA (position 1209-1223) |

B. Results. *E. coli* cells were stained with the five different probes described hereinabove and analyzed by flow cytometry as described in Example 1. The results are shown in TABLE 11.

TABLE 11

| Probes Used | Fluoresc. Intensity of Neg. Control | Fluoresc. Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| Probe 4 (SEQ I.D. No. 4) | 0.105 | 0.182 | N/A |
| Probe 8 (SEQ I.D. No. 8) | 0.107 | 0.107 | N/A |
| Probe 9 (SEQ I.D. No. 9) | 0.105 | 0.110 | N/A |

TABLE 11 (Cont.)

| Probes Used | Fluoresc. Intensity of Neg. Control | Fluoresc. Intensity of Pos. Sample | Signal to Noise |
|---|---|---|---|
| Probe 6 (SEQ I.D. No. 6) | 0.105 | 0.726 | 6.9 |
| Probe 7 (SEQ I.D. No. 7) | 0.106 | 1.074 | 10.1 |

The results shown in TABLE 11 clearly illustrate the specificity of the hybridization technique when used with PNA probes. The mammalian 28S rRNA probe and *S. aureus* probe did not cross-react to *E. coli* , while the 16S rRNA universal probe and the eubacterial probe hybridized as expected to *E. coli.*

Example 8. Comparison of DNA and PNA Probes Under Conditions Preferred by Each

A. Experimental Protocol. This experiment compared DNA and PNA probes in a *E. coli* system under both conditions optimized for DNA FISH and PNA FISH.. The EuB338 sequence was used to synthesize a DNA 18mer (SEQUENCE I.D. NO. 1 ) and a PNA 15mer (SEQUENCE I.D. NO. 7 ). Both the SSC buffer system and experimental protocol optimized for DNA probes (Example 1) and the PBS buffer system and experimental protocol optimized for PNA probe (Examples 3-5) were tested. The optimized PNA-FISH buffer system was composed of PBS and 0.5% SDS. The optimized SSC buffer system (which is conventional for DNA) was composed of 2X SSC, 50% formamide, 0.5% SDS and 100 µg/ml salmon sperm DNA.

B. Results. The data shown in TABLE 12 clearly demonstrate that the PNA probe (SEQUENCE ID NO. 7 ), whether in PBS or SSC buffer (conventional FISH buffer system), provided significant discrimination (signal-to-noise). Also, the signal was better in the optimized PBS buffer. The corresponding DNA probe (SEQUENCE ID NO. 3), however, showed significantly less (about 6-10 fold)

signal to noise. The DNA probe (SEQUENCE ID NO. 3) also performed better in the SSC buffer containing formamide as a denaturing agent, which was expected.

TABLE 12

| Samples | Fluoresc. Intensity of Neg.control | Fluoresc. Intensity of Pos.Sample | Signal to Noise |
|---|---|---|---|
| Probe 7 (SEQUENCE ID NO. 7) PNA-PBS buffer | 0.108 | 2.756 | 25.5 |
| Probe 7 (SEQ ID #7) PNA-SSC buffer | 0.131 | 1.964 | 15.0 |
| Probe 3 (SEQ ID #3) DNA-PBS buffer | 0.106 | 0.198 | 1.87 |
| Probe 3 (SEQ ID #3) DNA-SSC buffer | 0.110 | 0.250 | 2.27 |

Example 9. Detecting Bacteria inside Phygocytes

Images were taken using Metamorph software package (Universal Imaging Corporation, West Chester, PA) and Nikon epi-fluorescence microscope equipped with a mercury arc lamp and a cooled Star I CCD camera (Photometrics, Tucson, Arizona). The light source used was an HBO 100w HG mercury bulb (Fryer Co., Inc., Huntley, IL). Images, 576 x 384 pixels, were taken using a Nikon 100X oil immersion objective.

A. *In vitro* infection. Three (3) ml of 6% dextran was added to 10 ml of human blood obtained from a healthy donor and incubated at 37°C for 30 min. The PMN-rich layer then was collected. It was incubated with a preparation of *E. coli* previously prepared by incubating 10 ml of an *E. coli* solution (having an

absorbance of 0.1 at 600 nm wavelength, incubated for 30 min at 37°C, having the supernatant decanted and using the remaining layer of bacteria at the bottom of the flask) in PBS for 10 min at 37°C in a tissue culture flask. The cell monolayer was collected, washed with PBS and deposited onto microscopic slides using a Cytospin centrifuge at 800 x g for 5 min. Cells deposited on the slides were fixed with 4% paraformaldehyde for 15 min at room temperature and then stored in 70% ethanol at 4°C until use.

B. *In situ* hybridization on slides. Slides stored in 70% ethanol prepared as described hereinabove were rehydrated in PBS for 10 min. The slides then were treated with 1 µg/ml proteinase K in 20 mM TrisCl + 2mM $CaCl_2$ buffer for 15min at 37°C, followed by two washes with PBS. The slides were fixed again with 1% paraformaldehyde for 10 min followed by two washes with PBS. Five (5) pmol of probe 7 (SEQUENCE I.D. NO. 7) dissolved in PBS + 0.1% SDS was applied to each slide and covered with coverslips. Hybridization was carried out in a humidified box at 37°C for two hours. After hybridization, the slides were washed twice with PBS pre-warmed in 45°C water bath. The slides then were stained with 2.5 µg/ml Hoechst 33342 in PBS (Sigma, St. Louis, MO) for 5 min and washed once with PBS for 5 min. The slides then were mounted with VectaShield (Vector Laboratories, Burlingame, CA) and viewed under microscope.

C *In vivo* infection. Groups of CF-1 (outbred) mice (obtained from Charles River Laboratory, Wilmington, MA) were inoculated intraperitoneally with 1 x $10^6$ *E. coli*.. Blood from mice (approximately 1 ml)was collected by cardiac puncture at various times (5 min, 15 min, 1 hour, 2 hours, 3 hours, 6 hours, 8 hours and 12 hours) after inoculation. The blood was separated using 6% dextran gradient, washed and fixed as described in the above text. In situ hybridization was performed using Probe 7 (SEQUENCE I.D. NO. 7) as described above. PMNs isolated from infected mice were hybridized with fluorescein-labeled PNA probes targeting bacterial 16S rRNA. Fluorescein signal was collected with FITC visual filter cube for Nikon microscope (Fryer Co.Inc., Huntley, IL) with 480/30 nm exiciter filter, 505 DCLP dichroic mirror and 535/40 nm emitter filter.

D. Results. In both *in vitro* and *in vivo* systems, we have demonstrated that PNA-FISH technique described in this work can be used for diagnosis of bacteremia or sepsis. FIGURE 3 clearly shows that bacteria *E. coli* was brightly stained with FITC-labeled PNA probe (SEQUENCE I.D. NO. 7) six hours after the mice had

been inoculated with bacteria E. *coli*. Also, at the inoculum level of 1 x $10^6$ *E. coli* per mouse, bacteria in the bloodstream were detectedwith the PNA-FISH technique five minutes through 12 hours after inoculation, when the mice started to die. The PNA-FISH assay thus can be used for the identification of gram positive, gram negative bacterial or fungal infections, in a very short turn-around time (4-5 hours). This technique thus is very sensitive and provides valuble information to the physicians for the diagnosis of becteremia and sepsis.

Example 10. Morpholino Probes and their use in Fluorescent *In Situ* Hybridization and Solid Phase Capture

A. *In Situ* Hybridization. As described in Example 1, morpholino oligos can be used as probes for fluorescence in situ hybridization. A morpholino oligo (SEQUENDE I.D. NO. 4) is labeled with fluorescein at the amino-end of the peptide and is used to detect the complimentary sequence of 28S rRNA following the FISH assay as disclosed hereinabove in Example 1. The optimal morpholino hybridization conditions are determined as described in Examples 1, 2, 3, 4 and 5. EPICS®Profile II (Coulter Corp., Hialeah, Florida) flow cytometer, equipped with an Argon laser, is used to detect the probe. The laser is set to 15mW at 488nm, with fluorescein fluorescence acquired using the light selection filter of 525/30 nm (central wavelength/full bandwidth at half-maximal transmission).

B. Solid Phase Capture. Morpholino oligos (e.g., SEQUENCE I.D. NO. 4) can be affixed to a glass surface and complementary nucleic acid (DNA or RNA) detected as taught in U.S. patent application Serial No. 08/311462, previously incorporated herein by reference.

Bacteremia is a serious and urgent disease caused by the emergence and habitancy of various bacteria in blood. An individual may die within several hours to several days. Clinicians need rapid test results to confirm blood infection, to identify the invading organism and to administer an effective antibiotic to the individual. Currently, blood infections are tested using blood culture bottle method followed by gram stain. The current procedures take a day to a week, and sometimes even longer, for organisms that are hard to culture. Furthermore, false negative culture data can result in individuals who receive antibiotic therapy prior to or during sample collection.

Using the PNA-FISH technique we developed, we can detect bacteria in phagocytic cells in peripheral blood. Polymorphonuclear neutrophils (PMNs) form our first line of defense against invading bacteria. They serve as concentrators for bacteria. Fluorescently labeled peptide nucleic acid probes have been made targeting bacterial 16S rRNA. Using an *in situ* hybridization technique and PNAs (or morpholinos), bacteria inside PMNs can be brightly stained. This technique thus provides a rapid (4 hour) assay for detection of bacterial in test samples.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: YU, HONG
DUNN, DAVID A.
VALDIVIA, LYNN

(ii) TITLE OF INVENTION: POLYMERIC PEPTIDE PROBES AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ABBOTT LABORATORIES
(B) STREET: ONE ABBOTT PARK RD
(C) CITY: ABBOTT PARK
(D) STATE: IL
(E) COUNTRY: USA
(F) ZIP: 60064-3500

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 05/18/95
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: POREMBSKI, PRISCILLA E.
(B) REGISTRATION NUMBER: 33,207

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 708-937-6365
(B) TELEFAX: 708-938-2623

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(ix) FEATURE:
(A) NAME/KEY: 3' fluorescein
(B) LOCATION: 25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACCTTTTCTG GGGTCTGATG AGCGT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(ix) FEATURE:
(A) NAME/KEY: 3' fluorescein
(B) LOCATION: 25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGACAGCTTA TCATCGATAA GCTTT 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTGCCTCCC GTAGGAGT 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACCTTTTCTG GGGTC 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGGGGTCTT TTCCA 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTGCCTCCC GTAGG 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGCCTCCC GTAGG 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein

(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGCATCACA GACCT 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: 5' fluorescein
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTGGAGTAAC CTTTT 15

CLAIMS

1. An assay for detecting rRNA which may be present in a test sample, comprising:

a. contacting said test sample with a peptide nucleic acid (PNA) probe capable of attaching to said rRNA in said test sample conjugated to an indicator reagent comprising signal generating compound capable of generating a measurable signal;

b. detecting said measurable signal as an indication of the presence of rRNA in the test sample.

2. The assay of claim 1 further comprising performing said assay by flow cytometry.

3. The assay of claim 2 wherein quantitation is performed by exciting fluorescence and measuring said signal by using a light selection filter.

4. The assay of claim 3 wherein said signal generating compound is fluorescein.

5. The assay of claim 4 wherein said signal generating compound is rhodamine.

6. The assay of claim 3 further comprising fixing said test sample prior to performing step (a).

7. The assay of claim 6 further comprising hybridizing said test sample *in situ*.

8. An assay for detecting rRNA which may be present in a test sample, comprising:

a. contacting said test sample with a morpholino probe capable of attaching to said rRNA in said test sample conjugated to an indicator reagent comprising signal generating compound capable of generating a measurable signal;

b. detecting said measurable signal as an indication of the presence of rRNA in the test sample.

9. The assay of claim 8 further comprising performing said assay by flow cytometry.

10. The assay of claim 9 wherein quantitation is performed by exciting fluorescence and measuring said signal by using a light selection filter.

11. The assay of claim 10 wherein said signal generating compound is fluorescein.

12. In an fluorescence *in situ* hybridization assay for detecting the presence of rRNA which may be present in a test sample comprising the steps of (a) fixing said test sample, (b) hybridizing said rRNA in said test sample with a probe capable of attaching to said rRNA in said test sample conjugated to a signal generating compound capable of generating a measurable signal, and (c) detecting the presence of rRNA in said test sample by measuring the detectable signal, wherein the improvement comprises hybridizing said test sample with a peptide nucleic acid (PNA) or morpholino probe.

13. The fluorescence in situ hybridization assay of claim 12, wherein said signal generating compound is fluorescein.

14. A test kit for detecting the presence of rRNA which may be present in a test sample, comprising a container containing a peptide nucleic acid (PNA) or morpholino probe conjugated to a signal generating compound capable of generating a measurable signal.

15. The test kit of claim 13 wherein said signal generating compound is fluorescein.

16. An assay for detecting drug resistance genes which may be present in a test sample, comprising t

a. contacting said test sample which may contain drug resistance gene(s) with a peptide nucleic acid (PNA) probe or a morpholino probe capable of attaching to said drug resistance gene(s) in said test sample conjugated to an

indicator reagent comprising signal generating compound capable of generating a measurable signal; and

b. detecting said measurable signal as an indication of the presence of the drug resistance gene(s) in the test sample.

17. The assay of claim 16 performed by flow cytometry.

18. The assay of claim 17 wherein quantitation is performed by exciting fluorescence and measuring said signal by using a light selection filter.

19. The assay of claim 16 wherein said signal generating compound preferably is selected from the group consisting of fluorescein or rhodamine.

20. The assay of claim 19 wherein said drug resistance gene(s) in the test sample are fixed prior to performing the assay.

21. The assay of claim 19 further comprising hybridizing said test sample *in situ*.

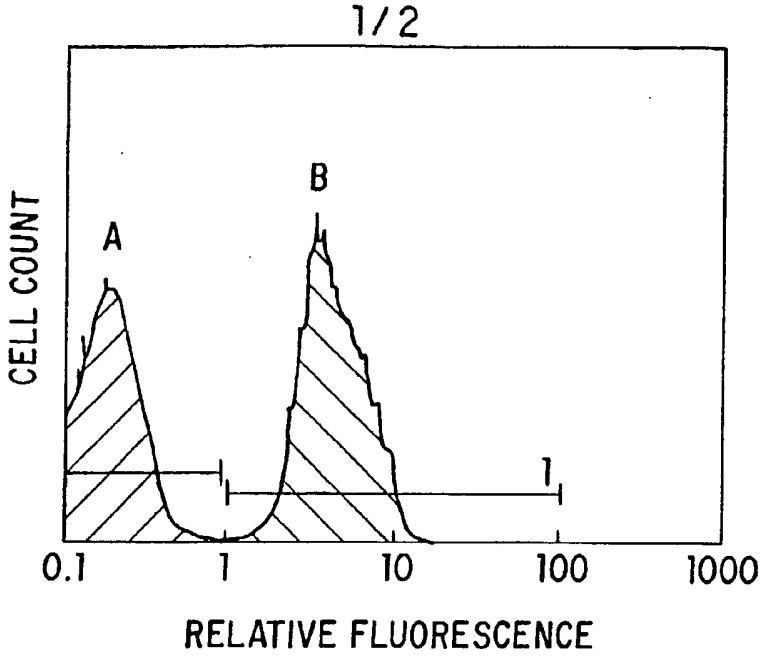


FIG. 1

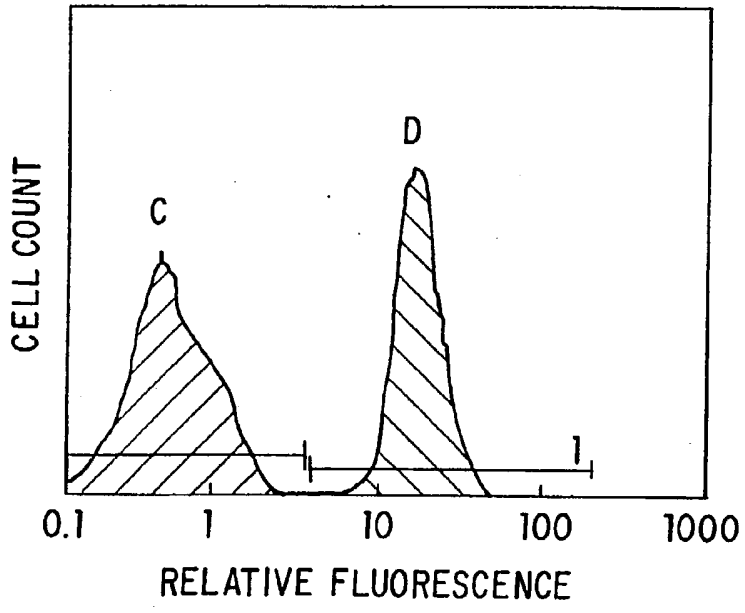


FIG. 2

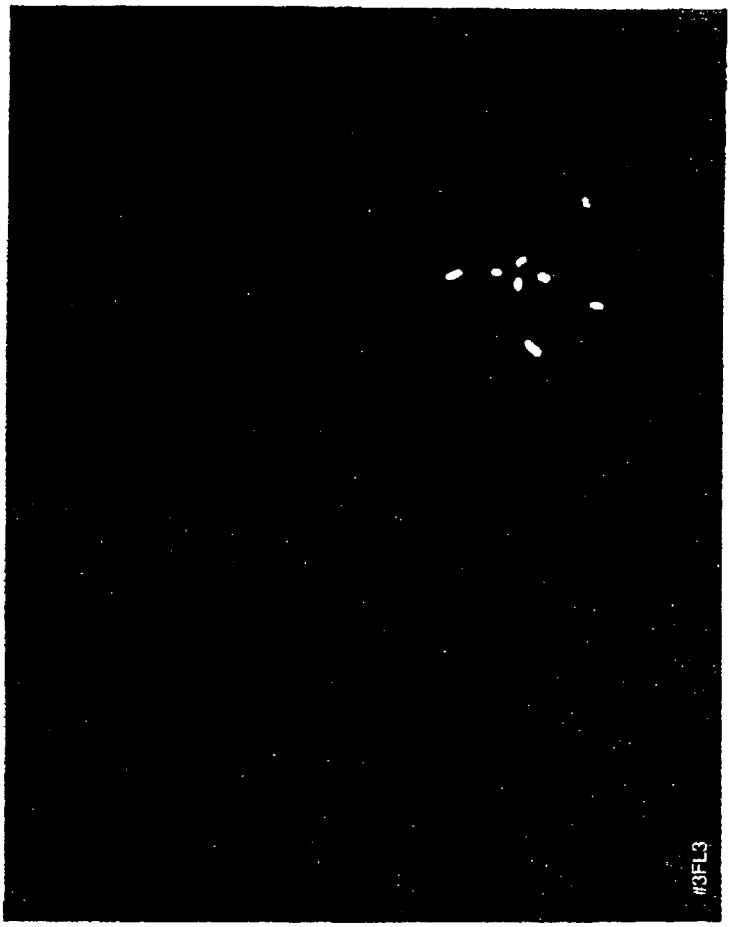


FIG.3

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCI/US 96/07075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 //C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,94 25477 (NIELSEN) 10 November 1994<br>see page 13, line 22 - line 25 | 1 |
| X | WO,A,93 24652 (CREMER) 9 December 1993<br>see page 8, line 22 - page 9, line 35;<br>claim 1 | 1-21 |
| X | WO,A,92 22647 (GENPHARM INTERNATIONAL INC.) 23 December 1992<br>see page 22, line 8 - line 11 | 1-21 |
| X | WO,A,95 08556 (AMERSHAM INT. PLC.) 30 March 1995<br>see page 2, line 6 - line 7 | 1-21 |
| Y | WO,A,90 10715 (MOLECULAR BIOSYSTEMS INC.) 20 September 1990<br>see page 7 | 1-21 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 10 September 1996 | 27.09.96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Osborne, H |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO,A,94 02642 (APROGENEX INC) 3 February 1994<br>see example 1<br>--- | 1-21 |
| Y | WO,A,94 02645 (RESEARCH DEVELOPMENT FOUNDATION) 3 February 1994<br>see example 7<br>--- | 1-21 |
| P,X | WO,A,95 32305 (DAKO) 30 November 1995<br>see the whole document<br>----- | 1 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 96/07075

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9425477 | 10-11-94 | US-A- | 5539082 | 23-07-96 |
| | | AU-A- | 6760494 | 21-11-94 |
| | | EP-A- | 0699208 | 06-03-96 |
| WO-A-9324652 | 09-12-93 | DE-A- | 4216949 | 02-12-93 |
| WO-A-9222647 | 23-12-92 | NONE | | |
| WO-A-9508556 | 30-03-95 | AU-A- | 7661794 | 10-04-95 |
| | | EP-A- | 0720615 | 10-07-96 |
| WO-A-9010715 | 20-09-90 | AU-B- | 646635 | 03-03-94 |
| | | AU-A- | 5289990 | 09-10-90 |
| | | CA-A- | 2011571 | 07-09-90 |
| | | EP-A- | 0462221 | 27-12-91 |
| | | JP-T- | 4505556 | 01-10-92 |
| WO-A-9402642 | 03-02-94 | AU-A- | 4681393 | 14-02-94 |
| | | AU-A- | 4775193 | 14-02-94 |
| | | EP-A- | 0662151 | 12-07-95 |
| | | WO-A- | 9402644 | 03-02-94 |
| WO-A-9402645 | 03-02-94 | AU-A- | 4775293 | 14-02-94 |
| | | CN-A- | 1083113 | 02-03-94 |
| WO-A-9532305 | 30-11-95 | AU-A- | 2522095 | 18-12-95 |